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Water fluxes through aquaporin-9 prime epithelial cells for rapid wound healing

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ABSTRACT

Cells move along surfaces both as single cells and multi-cellular units. Recent research points toward pivotal roles for water flux through aquaporins (AQPs) in single cell migration. Their expression is known to facilitate this process by promoting rapid shape changes. However, little is known about the impact on migrating epithelial sheets during wound healing and epithelial renewal. Here, we investigate and compare the effects of AQP9 on single cell and epithelial sheet migration. To achieve this, MDCK-1 cells stably expressing AQP9 were subjected to migration assessment. We found that AQP9 facilitated cell locomotion at both the single and multi-cellular level. Furthermore, we identified major differences in the monolayer integrity and cell size upon expression of AQP9 during epithelial sheet migration, indicating a rapid volume-regulatory mechanism. We suggest a novel mechanism for epithelial wound healing based on AQP-induced swelling and expansion of the monolayer.

1. Introduction

Cell migration is evident both for single cells and multi-cellular units. Despite distinct differences in the migration patterns, both these modes depend on a highly dynamic membrane, major cytoskeletal remodeling and cell–environmental interactions. Epithelial sheet migration is a common form of multi-cellular motility and is physiologically relevant at the skin and intestine during wound healing [1,2]. During this process, some cells at the wound margin polarize and form typical membrane protrusions associated with cell migration, such as lamellipodia and filopodia. As for single cells, small GTPases within the Rho family such as Rac, Cdc42 and Rho, are highly involved in the sheet migration. Rac inhibition of the three first cellular rows adjacent to the margin has for instance been shown to completely inhibit, whereas Rho was shown to have a major impact on the regularity of the sheet migration [3]. Moreover, it is highly influenced by the surrounding cells as well as mechanical forces, but it is unclear whether it is driven by: (i) cells at the margin pulling the sheet forward, (ii) cells behind pushing the sheet forward, or (iii) a combination of these processes. Furthermore, the cells in the epithelial sheet could be assigned different roles, e.g. a pioneer or a follower role, based on the expression of cell surface receptors and cell–cell contacts. Disturbing the order of the epithelial sheet by reducing cadherin cell–cell junction has been found to decrease the orientation within the migrating monolayer and thereby diminish the follower behavior [4]. Thus, a highly sophisticated interplay based on cellular characteristics such as cell–cell junction, migration promoting protein expression, location in the monolayer and the epithelial integrity is indeed required for epithelial sheet migration.

Recently, water fluxes through membrane-anchored aquaporins (AQPs) have been proposed to play a pivotal role in cell migration (reviewed in [5–7]). By their local appearance and abundance they could aid cell locomotion by facilitating shape changes like protrusion formation being required for migration into narrow spaces. There are 13 different mammalian AQPs known and this trait appears to be applicable on most of them. In brief, the mechanism is assumed to be based on an increase in the hydrostatic pressure causing the membrane to dislocate from the cytoskeletal anchorage and thereby forcing it to protrude outward. Furthermore, dilution of the gel-like cytoplasm and G-actin monomers should create a steep concentration gradient and facilitate diffusion of new actin monomers to the polymerizing actin filaments [5,6,8,9]. However, little is known about the effect of aquaporins in migration of a confluent epithelial sheet where para-cellular communication and cell-to-cell interactions via different cellular junctions are essential for monolayer tightness and epithelial integrity.

The aim of this study was to assess the effects of AQP9 on single cell and epithelial sheet migration. To achieve this, we used the canine kidney cell line MDCK-1, as a model system. Here, we stably

Abbreviations: AQP, aquaporin; CMV, cytomegalovirus; PFA, paraformaldehyde; NA, numerical aperture; GFP, green fluorescent protein; EGF, epidermal growth factor.

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expressed GFP-AQP9 under regulation of a CMV promoter yielding a relatively high expression. They were then subjected to single cell and epithelial sheet motility assays.

Our findings suggest that increased water fluxes promote both single cell and epithelial sheet migration. Moreover, we provide evidence for a novel mechanism of wound healing based on swelling and expansion of the monolayer.

2. Materials and methods

2.1. Preparation of stable AQP9-GFP expression in MDCK-1 cells

Cells were cultured in Dulbeccos Modified Eagles Medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 U/ml penicillin, 1 mM Sodium Pyruvate and 2 mmol L-glutamine (all obtained from GIBCO BRL/Invitrogen Carlsbad, CA, USA). cDNA encoding for AQP9 was prepared as described in [10] and cloned into the retroviral backbone pRetroQ-AcGFP1c1 (Clontech laboratory Inc, Mountain View, CA). Retroviral particles containing GFP-AQP9 with pA10 trophism were produced in the packaging cell line GP-2 293 (Clontech) for 48 h. The MDCK-1 cells were then incubated with the virus-containing supernatant for 24 h and subsequently cultured in medium containing 4 μg/ml puromycin (Sigma–Aldrich, St Louise, MO). GFP-expressing cells were further sorted according to GFP fluorescence in an ARIA III cell sorter (Becton, Dickinson, Franklin Lakes, NJ). After generation of stable cell lines, they were constantly passaged simultaneously.

2.2. Imaging

For structured illumination confocal imaging and for high resolution epi-fluorescent imaging an Axiovert 200 M (Zeiss, Jena, Germany) stage equipped with a structured illumination-aperture correlation unit (VivaTome, Zeiss) and 63× (NA 1.25; Zeiss) and 40× (NA 1.3; Zeiss) objectives, Axiocam Mrm CCD camera and a HXP 120 C fluorescent lamp. For laser scanning confocal imaging an Axio Observer Z1 stage was used equipped with a LSM700 (Zeiss) confocal unit and 20× (NA 0.8; Zeiss) objective. For wound healing assays, the cells were imaged on an inverted bench top JuLI stage (Saveen & Werner, Digital Bio, Seoul, Korea) inside the incubator at 5% CO_2. To visualize single cell migration, a Nikon Ti-E staged with Perfect Focus and a 20× objective was used (Nikon, Tokyo, Japan). This system also contained a pE-2 lamp (CoolLED, Andover, UK), a micro-incubation chamber (QE-1), a TC-344B Heat Controller (both purchased from Warner Instruments, Boston, MA) and a Clara interlined CCD (Andor, Belfast, UK).

2.3. Wound healing

The cells (1–3 × 10^5 cells/ml) were seeded into the two compartments of an Ibidi Wound healing chamber (Ibidi, Martinsried, Germany). The cells were allowed to grow under regular culturing conditions for 3–5 d. After this, the insert was removed with a tweezer yielding a standardized wound of 500 μm. The dish was washed and subsequently imaged in serum-free medium for 10 h. Images were acquired sequentially every 5 min. The images were subsequently assessed with the wound healing plug-in in the open source software Cell Profiler [11]. Individual thresholds were set to the different image sequences. To analyze the number of cells/field of view the cells were fixed in 4% paraformaldehyde (PFA, Sigma–Aldrich) 4 d after seeding. Fixation was performed before and 1–7 h after removing the wound healing insert. They were then washed in PBS and subsequently mounted in ProLong Gold containing DAPI (Molecular probes/Invitrogen, Carlsbad, CA). Confocal images were captured at the epithelial monolayer with a 40× (NA 1.3; Zeiss) objective and the number of nuclei/field of view were counted manually in ImageJ.

2.4. Actin staining

To stain the actin cytoskeleton, the cells were fixed for 20 min at room temperature (RT) in 4% PFA. They were then washed in PBS, permeabilized in 0.1% Triton X-100 (Sigma–Aldrich) and washed again. Phalloidin-conjugated Alexa 568 (1:200; Molecular probes) was added to the samples that were subsequently incubated for 45 min at RT. Following a final wash, the cells were mounted in ProLong Gold containing DAPI.

2.5. Single cell migration

The cells were seeded in glass-bottom culture dishes (MatTek Corporation, Ashland, MA) starved and allowed to adhere for 10–12 h. One hour before acquisition the medium was changed to imaging medium previously described in [12] and stimulated with 50 nM epidermal growth factor (EGF). Images were then acquired every 30 s for 7 h in both bright field and epi-fluorescence. The image sequences were subjected for analysis in the image processing software Imaris using the "Imaris track" module. In brief, a binary mask was created and the cells were subsequently tracked over time based on the center of the cell mass. Only motile single cells that were inside the field of view throughout image acquisition were subjected for analysis. The coordinates obtained from the tracking procedure were the loaded into Ibidis chemotaxis and migration tool (Ibidi) and the Euclidean and accumulated distances were calculated.

2.6. Proliferation rate

The cells were seeded at 2 × 10^5 cells/ml in culture dishes and then placed on the JuLI bench top microscope inside the incubator. Image acquisition was carried out at an interval of 10 min for 100 h. The cells were subsequently tracked over time in ImageJ and the time between two divisions was calculated.

2.7. Transepithelial electrical resistance (TER)

The cells were seeded at a density of 1–3 × 10^5 cells/ml on collagen-coated Transwell filters (pore size 3 μm, Corning, New York, NY). The TER of the monolayer was measured 1–6 d later with a volt-ohmmeter (World Precision Instruments, Sarasota, FL).

2.8. Statistical analyses

All data is presented as mean ± SEM. Statistical analyses are based on two tailed, non-parametric Mann–Whitney tests. The significance was rated $*$ when $0.05 > p < 0.01$, $**$ when $0.01 > p < 0.001$ and $***$ when $p < 0.001$.

3. Results and discussion

3.1. Cells expressing AQP9 show increased single cell motility but migrates within smaller areas

The effect of AQP9 on the motility of MDCK-1 cells was assessed by generation of a stable cell line expressing GFP-AQP9. Here, AQP9 was shown to localize at the plasma membrane. Furthermore, to analyze the relation between the localization of AQP9 and actin, intensity profile plots of these molecules labeled with spectrally separated fluorophores was measured. Indeed, the intensity fluctuations between GFP-AQP9 and actin correlated while no correla-